taining chloramine T (100 µL) was added to each well except those designated as blanks. The same amount of oxidation buffer without chloramine T was added to each blank well. The plate was mixed by gentle shaking and left for 5 min. Ehrlich's reagent (100 µL) was added to each well and mixed thoroughly. The plate was covered with an adhesive plate seal and incubated in a water bath at 60°C for 45 min. Absorbance was read at 570 nm using an MRX microplate reader (Dynatech Laboratories, West Sussex, UK). The average optical densities (OD) for the calibrators and unknown samples were corrected for the average blank OD by subtraction. A calibration curve (Figure 2) was constructed, and the hydroxyproline concentration of the unknown samples was calculated from the "best-fit" equation of the line.

The within-run CV was 4.3%, 3.8%, and 4.1% for the concentration ranges, 2–4.5, 5–8, and 8–10 µg/mL hydroxyproline, respectively. The interassay CV in the lower range was 9.3% and was calculated from 16 runs with repeated hydrolysis. The sensitivity of the assay was determined as the concentration at which the standard deviation of the result was half of that result. Using this criterion, the sensitivity of the assay is 0.1 µg/mL hydroxyproline, and it is linear up to 40 µg/mL. The recovery of added hydroxyproline was 100 ± 8% (range 88%–116%), and the recovery of added gelatin was 93 ± 12% (range 75%–115%) based on a nominal hydroxyproline content.

This assay for hydroxyproline in connective tissue hydrolysates shows an improved reproducibility compared with the automated technique described by Worsfold et al. (5), which has intra- and interassay CVs of 5.9% and 12%, respectively (unpublished data), although those statistics were compiled over several years of urine assays that have variable blank optical densities. The procedure is convenient and allows increased sample throughput using minimum sample and reagent volumes. The equipment used is nowadays more widely available than segmented flow analyzers. The method as described did not work well for urines, which contain less hydroxyproline and would not be diluted after hydrolysis, so that larger amounts of hydrochloric acid and sodium chloride are carried forward into the assay.

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Use of DAPI Staining for Quantitation of Cell Chemotaxis

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Cell migration is a fundamental biological phenomenon that plays a pivotal role in developmental differentiation, inflammatory responses, wound healing, and tumor metastasis. Cells migrate in response to concentration gradients of diverse protein growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor, as well as lipid mediators such as sphingosine 1-phosphate (SPP), lysophosphatidic acid, and arachidonic acid. The ability of cells to react chemotactically to stimuli is of particular importance in angiogenesis, the process whereby new blood vessels are formed (2).

A variety of methods exist by which the migratory behavior of cells can be evaluated. These methods include labeling of live cells with fluorescent and luminescent tags (5,13), the use of cell type-specific stains (11), or the use of broad specificity stains such as hematoxylin-eosin mixture (9). Some of these methods are complex and require specialized genetic constructions or equipment, while others are time consuming and cumbersome.

Our current investigations have focused on the characterization of chemotactic responses of endothelial cells and smooth muscle cells to clarify the essential aspects of angiogenic responses. These two cell types are intimately involved in formation of capillaries and mature vessels (1,3,6,7). The migration of these cells toward their specific chemoattractants, SPP and PDGF, respectively, is routinely measured by counting cells that traverse the filter separating the upper compartment of a chemotactic chamber from the bottom well containing the stimulus. The cells that reach the lower surface of the filter are usually stained with hematoxylin and eosin and enumerated microscopically.

Although the hematoxylin-eosin staining is technically simple, the process is rather cumbersome and requires many steps to complete (Table 1). More importantly, when a vigorous chemotactic response is quantified, the cells of interest are not easy to enumerate accurately because of difficulties involved in determining the boundaries between closely adjacent cells and because of the irregular shape of the cells. We have developed a simple and rapid method to overcome these difficulties by employing a 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) staining technique. DAPI intercalates

into the minor groove of AT-rich regions of chromosomal DNA and emits strong blue fluorescence upon illumination with UV light (8). As a result, the nuclei of stained cells clearly stand out from a darker blue background (Figure 1).

Table 1 demonstrates the simplicity of the DAPI procedure. Instead of a series of staining, rinsing, and washing steps necessary for hematoxylin staining, only a single step is needed for the DAPI method. A small drop of DAPI solution (5 µg/mL) is applied to the filter, and after a brief incubation in the dark, the cells are counted under a fluorescence microscope, the time not being a critical factor. The amount of DAPI is more than sufficient to saturate the available DNA, and there is no apparent difference in staining intensity obtained with staining solutions of 1 or 5 µg/mL (not shown).

Figure 1 shows a side-by-side comparison of DAPI and hematoxylin staining of human smooth muscle cells. At 20× magnification (Figure 1, panels A and B), numerous bright nuclei are observed in the DAPI-stained cells. Although hematoxylin-stained nuclei are visible in Figure 1, panel A, the counting of these nuclei is difficult because of crowding of the field (a problem when high levels of migration are observed) and because of the cells overlapping each other, thereby making their outlines indistinct. The advantage of the DAPI method is even more obvious when comparing Figure 1, panels C and D (50× objective). Whereas the hematoxylin dye adequately visualizes the cells (Figure 1, panel C), it is difficult to determine where one cell ends and another begins; thus, accurate enumeration is a laborious task and not easily amenable to automated analysis. This problem is completely obviated by DAPI staining (Figure 1, panel D), which results in visualization of distinct, clearly defined nuclei. Note that panels C and D in Figure 1 are matched and aligned. After DAPI staining, the filters were processed by the hematoxylin method to emphasize the striking difference in the ease of analysis between the two procedures. Very similar results were obtained with rat smooth muscle cells (not shown) and with bovine pulmonary aortic endothelial cells (Figure 2). The DAPI staining procedure ren-

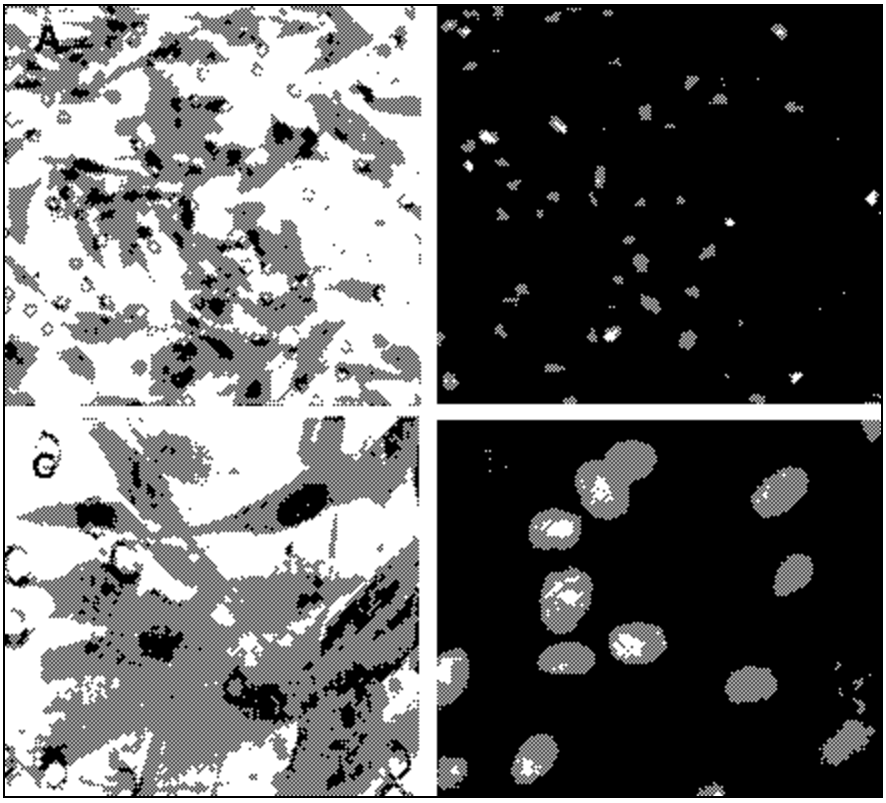

Figure 1. DAPI and hematoxylin staining of human aortic smooth muscle cells. Cultures of cells obtained from Cell Systems (Seattle, WA, USA) were grown in T75 tissue culture flasks in 10% fetal bovine serum-DMEM (Life Technologies, Rockville, MD, USA) to approximately 90% confluence, harvested, and used in chemotactic assays as described (4). Platelet-derived growth factor BB (Calbiochem-Novabiochem, San Diego, CA, USA) was used as chemoattractant at a concentration of 50 ng/mL. After 6 h of incubation, the cells that migrated through and adhered to the bottom surface of the filters were fixed in formaldehyde and stained as described in Table 1. Cells were photographed under 20× magnification (panels A and B) or 50× magnification (panels C and D). In panels A and C, the hematoxylin-stained cells are shown; panels B and D present DAPI-stained nuclei. Filter D was developed with DAPI and photographed using fluorescence microscope. The filter was then stained with hematoxylin (panel C) to demonstrate the close correspondence of the two methods.

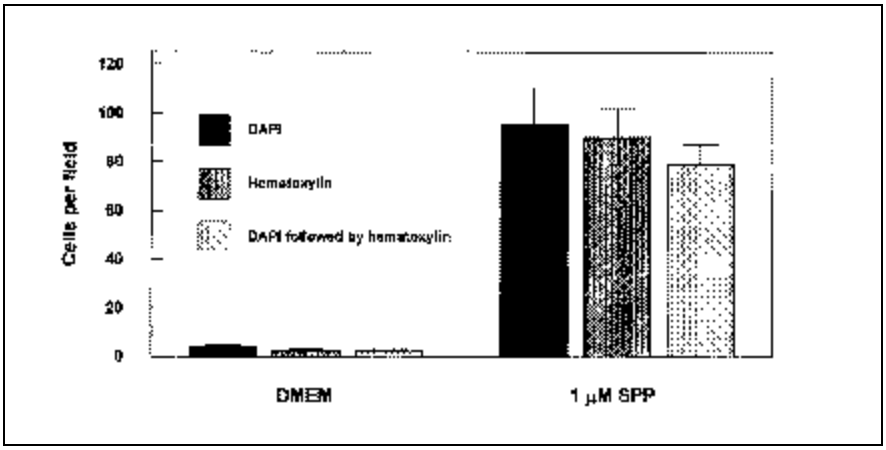

Figure 2. DAPI and hematoxylin staining of bovine pulmonary aortic endothelial cells. Cultures of cells (Clonetics, San Diego, CA, USA) were grown, and chemotaxis assays were performed as described (4,15). SPP (Calbiochem-Novabiochem) was used as a chemoattractant (1 µM). Following migration, the cells were stained with DAPI or hematoxylin and counted; the filters that had been stained with DAPI were subsequently developed with hematoxylin and recounted. It should be noted that DAPI staining cannot follow hematoxylin treatment because of irreversible denaturation of the DNA; however, the reverse order is acceptable.

Table 1. Comparison of Hematoxylin and DAPI Staining Techniques

Hematoxylin	DAPI
Stain with hematoxylin	
Rinse with water	
Dip in 70% ethanol	
Dip in $(NH_4)_2SO_4/NaHCO_3$ solution	
Dehydrate in 100% ethanol	
Counter-stain with eosin	
Rinse with water	Stain with DAPI
Count	Count

Details of hematoxylin staining method have been described previously (9). Stock solution of DAPI in water (10 mg/mL) is aliquoted and kept frozen at -20°C. The stock is diluted to 5 µg/mL in PBS, and sufficient volume is used to cover the surface of Transwell® (Fisher Scientific, Pittsburgh, PA, USA) filter containing cells that have been fixed with 5% formaldehyde solution. After brief incubation in the dark, the DAPI-stained cells are illuminated with UV light and visualized under a 20× or 50× objective in an Olympus BH-2 (Olympus America, Melville, NY, USA). The DAPI fluorescence persists for long periods of time, and the filters can be stored for several days without any degradation of the signal.

ders images amenable to digital scanning and counting with any of the commercially available combinations of a digital camera and scanning software such as SigmaScan™ (Jandel Scientific, San Rafael, CA, USA). Examples of the use of DAPI staining and image cytometry include the analysis of bacterial nucleoids (14), human peripheral blood lymphocytes and breast carcinoma cells (10), and interphase cell nuclei (12).

In addition to the visualization by the two methods, we also compared the efficiency of counting of cells stained by either of the two procedures. Bovine endothelial cells were allowed to migrate toward SPP, fixed with formaldehyde, and stained. As shown in Figure 2, each technique yields comparable numbers, with the double staining resulting in a somewhat diminished count, presumably caused by detachment of a few cells from the filter during the second round of staining.

This communication demonstrates that the DAPI method of quantitative analysis of migration is applicable to cells as diverse as human and rat smooth muscle and bovine endothelial cells. The method has the advantage of being rapid, inexpensive, and sensitive. Most importantly, the improved accuracy of cell enumeration based on staining of cell nuclei removes a major uncertainty from the quantitative analysis of chemotaxis. The technique can be easily combined with commonly used digital analysis systems.

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